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14. ABSTRACT

Prostate carcinogenesis is a multi-step process resulting in the transformation of prostatic epithelial cells into invasive carcinoma and metastasis. In recent years, mouse models have emerged that recapitulate salient features of prostate carcinogenesis found in human disease. These models illuminate the molecular events that result in transformation and disease progression. In addition, mouse models can be used to identify molecular targets and test chemotherapeutic agents that may alter the course of disease. We have generated a new mouse model to further delineate targets are critical for cancer progression. This genetically engineered mouse is a cross between the TRAMP and PSCA-TVA transgenic mice. The resultant TRAMP-TVA breed develops prostate cancer epithelial cells that express a unique avian viral receptor (TVA). TRAMP-TVA mice are susceptible to infection by avian sarcoma leucosis viruses (ASLV) that carry specific genetic material that can target intracellular pathways. The prostate cells of these mice, therefore, are targets for specific gene transfer of imaging genes and small hairpin nuclear RNAs (shRNAs) resulting in knockdown of specific targets. TRAMP-TVA mice demonstrate PIN lesions at 8 weeks and develop adenocarcinoma at 6-15 months. We have been able to demonstrate PSCA-driven expression of the TVA viral receptor in these lesions. Orthotopic and intraperitoneal injection of virus containing the luciferase gene results in luminescence signal from infected prostate epithelial cells. Further development of this model will enable the effect of genetic alterations targeted to the prostate to be evaluated in the setting of an in vivo metastatic prostate cancer model.

15. SUBJECT TERMS

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INTRODUCTION:

Prostate carcinogenesis is a multi-step process resulting in the transformation of prostatic epithelial cells into invasive carcinoma and metastasis. In recent years, mouse models have emerged that recapitulate salient features of prostate carcinogenesis found in human disease. These models illuminate the molecular events that result in transformation and disease progression. In addition, mouse models can be used to identify molecular targets and test chemotherapeutic agents that may alter the course of disease. We have generated a new mouse model to further delineate targets are critical for cancer progression. This genetically engineered mouse is a cross between the TRAMP and PSCA-TVA transgenic mice. The resultant TRAMP-TVA breed develops prostate cancer epithelial cells that express a unique avian viral receptor (TVA). TRAMP-TVA mice are susceptible to infection by avian sarcoma leucosis viruses (ASLV) that carry specific genetic material that can target intracellular pathways. The prostate cells of these mice, therefore, are targets for gene transfer of imaging genes and small hairpin nuclear RNAs (shRNAs) resulting in knockdown of specific targets. TRAMP-TVA mice demonstrate PIN lesions at 8 weeks and develop adenocarcinoma at 6-15 months. We have been able to demonstrate PSCA-driven expression of the TVA viral receptor in these lesions. Orthotopic and intraperitoneal injection of virus containing the luciferase gene results in luminescence signal from infected prostate epithelial cells. Further development of this model will enable the effect of genetic alterations targeted to the prostate to be evaluated in the setting of an in vivo metastatic prostate cancer model. This approach will facilitate targeted genetic events in prostate epithelial cells without the need for crossbreeding.

BODY:

The overall goal of this proposal is to utilize an established, well-characterized mouse model of prostate cancer to further delineate molecular targets that may effect cancer progression and metastasis. In order to rapidly evaluate a variety of select target genes, we created a new transgenic mouse (TRAMP-TVA). The concept of the TRAMP-TVA mouse model is shown in Figure 1. We have previously generated a PSCA-TVA mouse that utilizes the PSCA promoter to drive expression of the avian viral receptor, TVA, in prostate epithelial cells. This receptor is required for efficient gene transfer of avian viral vectors (RCAS) that are genetically engineered to express imaging genes, oncogenes, or shRNAs. The advantages of the TVA system include stability of gene transfer (viral genes integrate into host genome) and the ability to introduce multiple genetic changes into a single cell via multiple rounds of infection.

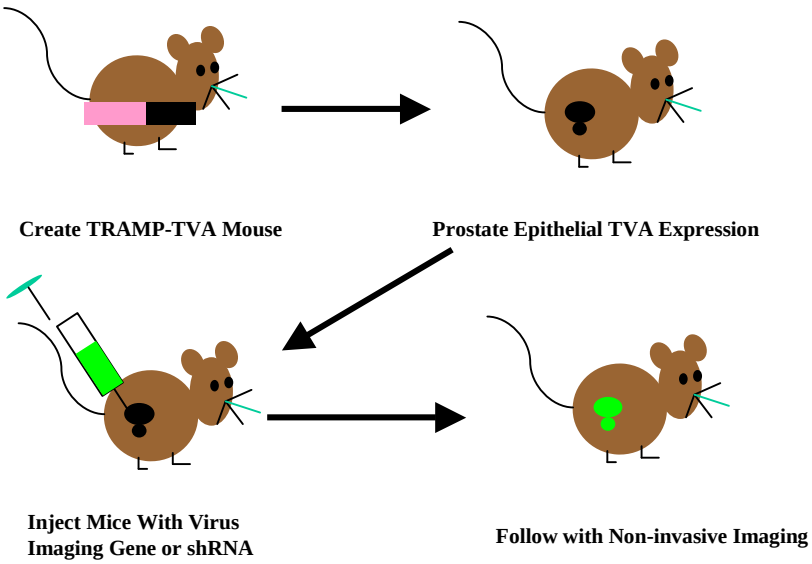


Figure 1: Viral-mediated gene transfer of TRAMP-TVA transgenic mouse. The TRAMP-TVA mice express the TVA avian viral receptor in prostate tissue under the direction of the PSCA promoter. Genetically engineered avian viruses containing the luciferase imaging gene and/or shRNA directed at specific target genes are intraperitoneally or orthotopically injected. Mice are imaged for reporter gene expression with change in tumor development/progression over time.

The TRAMP mouse is a well-established mouse model that utilizes the prostate-specific probasin promoter to drive expression of the SV40 large T antigen, resulting in PIN, adenocarcinoma, and eventually, metastatic disease in male mice(1). Previous work has demonstrated increased PSCA expression in prostate cancer cells from TRAMP mice(2). Therefore, we predicted that prostate cancer cells from TRAMP-TVA mice would express high levels of the TVA receptor. The creation of TRAMP-TVA mice should enable transfer of genetic information via avian viruses in order to study the effect of shRNA on disease initiation and progression. Combined transfer of imaging genes (luciferase and/or GFP) will allow this process to be followed non-invasively over time. The specific aims of this project are listed below:

AIM 1: Establish a TRAMP-TVA transgenic line with characterization of prostate tumorigenesis as well as TVA expression within prostate cells

AIM 2: Non-invasive imaging of tumor development and progression in TRAMP-TVA mice

AIM 3: Demonstration of viral mediated shRNA delivery to TRAMP-TVA mice cancer cells

Tasks Involved in Accomplishing Aim 1:

1) Cross-breeding of TRAMP and PSCA-TVA mice
2) Confirmation of TVA expression and prostate tumor development in mice generated from the TRAMP-TVA cross

Materials/ Methods/Outcomes (Aim 1):

PSCA-TVA Mice: We have demonstrated that PSCA-TVA mice express the TVA receptor in prostate epithelial cells. Both basal and luminal cells exhibit TVA expression (Fig. 2). Additionally, we have observed that TVA expression is restricted to prostate and bladder epithelial cells, and stomach mucosal cells.

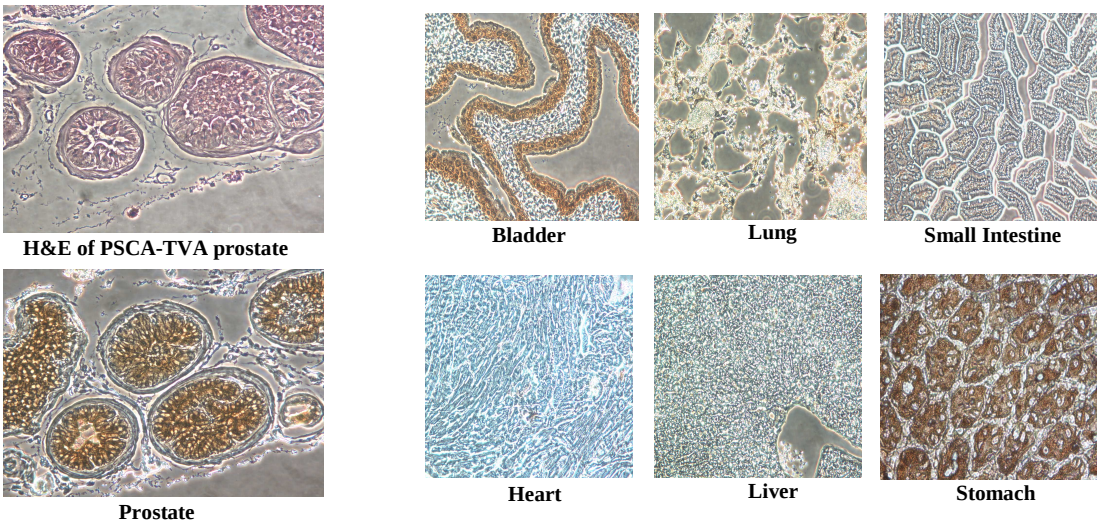


Figure 2: Normal tissue distribution of TVA in PSCA-TVA transgenic mice. Multiple stable transgenic lines were screened with equivalent results. A polyclonal antibody against TVA was used for immunostaining or paraffin-embedded tissues. H&E of the prostate is shown on the left. TVA expression is restricted to the prostate, bladder, and stomach of male mice.

Viral-Mediated Gene Delivery Into PSCA-TVA Mouse Prostate: In order to confirm prostate epithelial cells are the targets for RCAS infection, virus containing polyoma virus middle T antigen (MT) was injected orthotopically into the dorsal prostatic lobe of PSCA-TVA mice. In situ hybridization was performed 3 months post infection. The majority of epithelial cells within the dorsal lobe of the prostate where injection occurred were positive for MT antigen gene expression. This experiment confirms that PSCA-TVA mice are susceptible to efficient and targeted gene transfer.

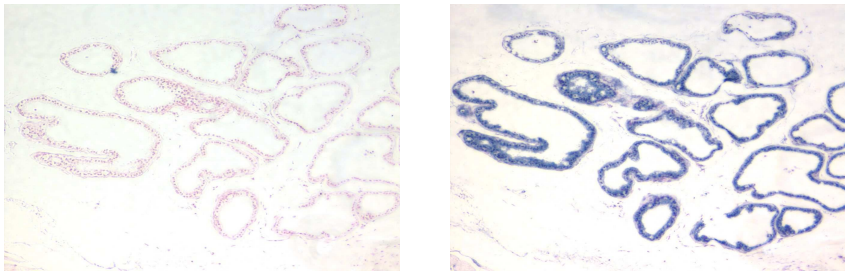


Figure 3: PSCA-TVA mice infected with the Polyoma MT antigen are analyzed for MT expression via in situ hybridization. The prostate epithelial cells show efficient infection and expression of the MT oncogene.

Imaging of PSCA-TVA Mice: DF-1 viral producing cells were transfected with an RCAS viral vector containing the luciferase gene (RCAS-Luc). A portion of cells was harvested after two weeks of culture and passage. Strong luciferase activity was observed in cell lysates, which confirmed that the DF-1 cells were producing functional virus (data not shown). Subsequently, PSCA-TVA transgenic mice and WT controls were injected orthotopically with DF-1 cells producing RCAS-Luc. The mice were imaged with the CCD camera 2 weeks after injection to allow time for the viral producing cells to be cleared by the mouse immune system. The PSCA-TVA transgenic mice showed luminescence in the pelvis, indicative of infection by RCAS-Luc. The WT mice, on the other hand, did not acquire luminescence. All PSCA-TVA mice injected with RCAS-Luc were successfully infected, as reflected by pelvic luminescence, indicating efficient viral infection.

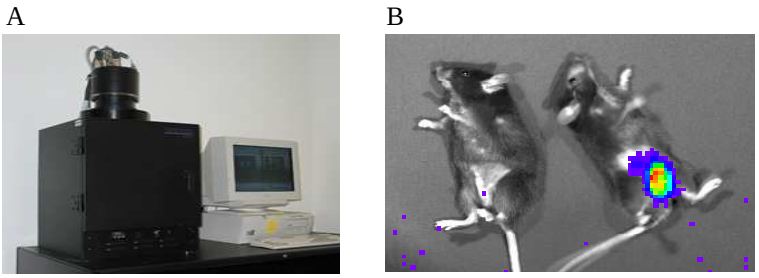


Figure 4: Imaging of WT and PSCA-TVA transgenic mice with the CCD camera post-injection with RCAS-luciferase. The CCD camera is pictured in A. WT and PSCA-TVA mice are photographed with the CCD camera two weeks post-injection in B. Only PSCA-TVA mice are infected with virus and show stable luciferase signal.

Characterization of TRAMP-TVA mice: We examined males generated from TRAMP-TVA crossbred mice for development of PIN and progression to prostate adenocarcinoma (Fig. 4). Mice were genotyped for the presence of the TVA gene and SV40 large T antigen via PCR (data not shown). TRAMP-TVA mice were sacrificed at various timepoints to determine cancer initiation and progression. Immunohistochemistry with anti-TVA antibodies confirmed the presence of the TVA receptor expression in PIN lesions and well-differentiated adenocarcinoma of TRAMP-TVA mice. Ki-67 activity was markedly increased in the TRAMP-TVA mice with PIN. Proliferative activity is an important factor for efficient viral infection, since the avian retroviruses require cellular proliferation for infection.

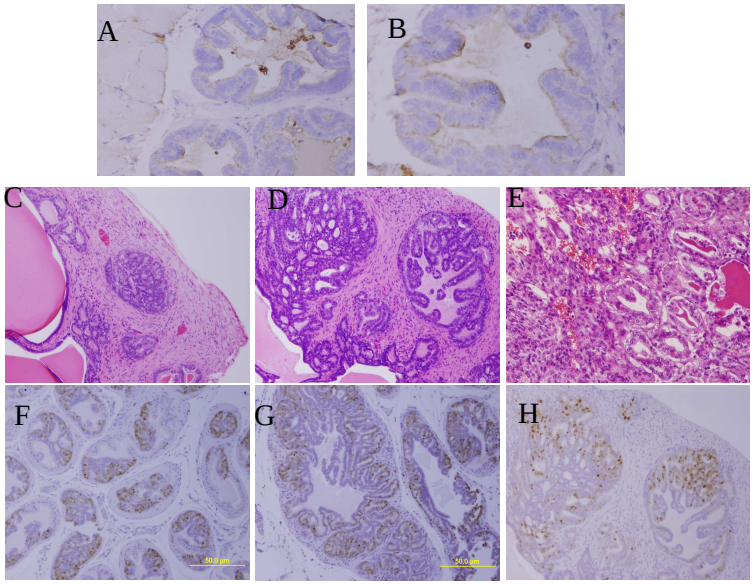


Figure 5: Prostate Epithelial Cell TVA Expression and Tumor Development and Progression in TRAMP-TVA Mice. TRAMP-TVA crossbred mice were confirmed by tissue genotyping and males were sacrificed at 8 weeks to determine TVA expression. The TVA viral receptor was demonstrated by immunohistochemistry on the apical surface of TRAMP-TVA mice (A&B). TRAMP-TVA mice develop PIN lesions by 8 weeks, as shown on H&E sections and with increased Ki-67 staining (C, F). These lesions progress to well-differentiated (D,G, H) and poorly differentiated adenocarcinoma (E) at 4 months and 12-15 months, respectively.

Summary Aim 1: We successfully crossbred PSCA-TVA mice with TRAMP mice and demonstrated that these mice develop prostate cancer, as expected. The PIN and cancer lesions express TVA, indicating that these cells are capable of targeted infection by RCAS viruses introduced orthotopically or intraperitoneally.

Tasks Involved in Accomplishing Aim 2:

1) Infection of TRAMP-TVA prostate epithelial cells with viral vectors containing luciferase at various timepoints in development
2) Imaging of infected TRAMP-TVA mice with charged coupled device (CCD) camera over time

Materials/Methods/Outcomes (Aim 2):

Infection and Imaging of TRAMP-TVA Mice: As described above, orthotopic injection of avian virus containing luciferase is capable in infecting the prostate of PSCA-TVA mice. Intraperitoneal injection (IP) is a preferred approach for viral gene delivery since is less invasive than orthotopic injection and systemic distribution of the virus may enable metastases to be targeted. In order to perform IP injections, we concentrated the RCAS virus via ultracentrifugation and performed viral tittering experiments to confirm adequate infectivity. Previous reports indicated that 2-day old mice were the most susceptible to IP infection, therefore, we performed a pilot experiment on 2-day old PSCA_TVA pups to determine whether or not

targeting to the prostate could be achieved. The mice were imaged at 1 week and 1 month post- injection. The results of the initial IP experiments are shown in Figure 6. The mice demonstrated pelvic luminescence in the proximity of the prostate gland.

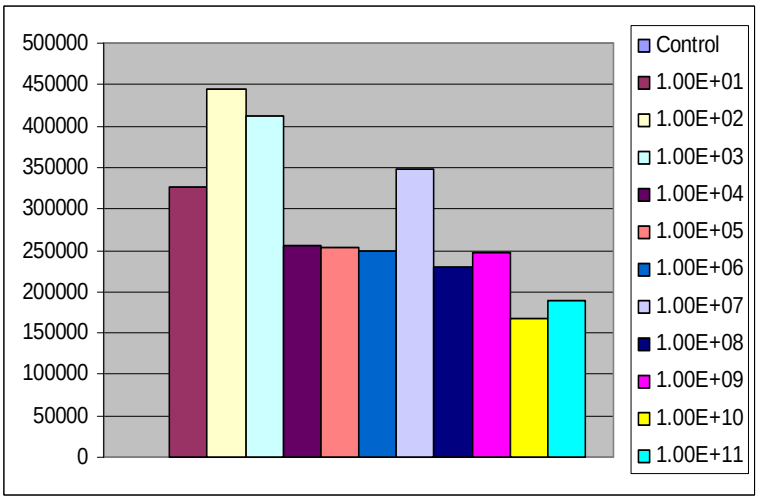
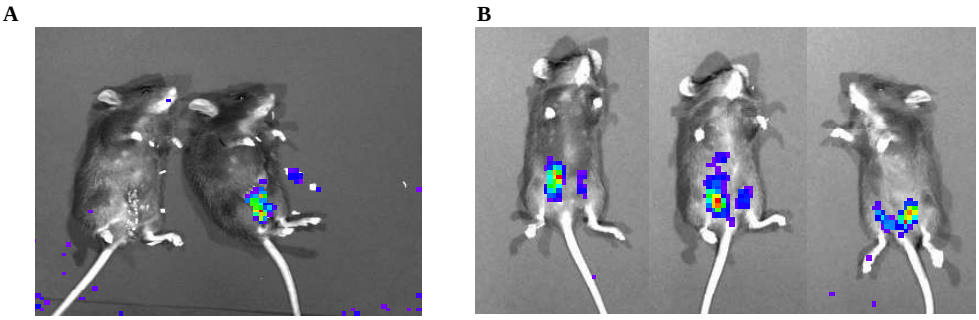


Figure 6. IP delivery of ASLV-luciferase virus. Graph demonstrates production of high titer virus from the supernatent of DF-1 viral producing cells infected with RCAS-luciferase. WT and TVA+ Mice infected at 2-days of age and imaged at 1 month of age. Durable pelvic luminescence is observed exclusively in TVA+ mice.



WT and TVA+ TVA+

In order to evaluate imaging gene transfer into prostate tissue of TRAMP-TVA mice, we attempted IP injections at 2-days, 1 month, and 3 months of age. The IP injections were performed daily for 7 days. Mice were imaged with the CCD camera following the last day of injection (data not shown). CCD imaging 4-months after the initial injection demonstrated durable prostatic luciferase expression (Figure 7A). Pelvic exploration and prostate exposure confirmed all signal was being emitted from infected prostatic tissue (Figure 7B). When IP injections were performed in mice over the age of one month, we were not able to observe luciferase expression, despite escalation in viral titer and increased number of injections (data not shown). The reason for the inability to demonstrate efficient gene transfer in older mice could be due to the mature immune system resulting in neutralization of virus when given IP. Therefore, IP injection may not be feasible in immunocompetent mice that are more than a few days old.

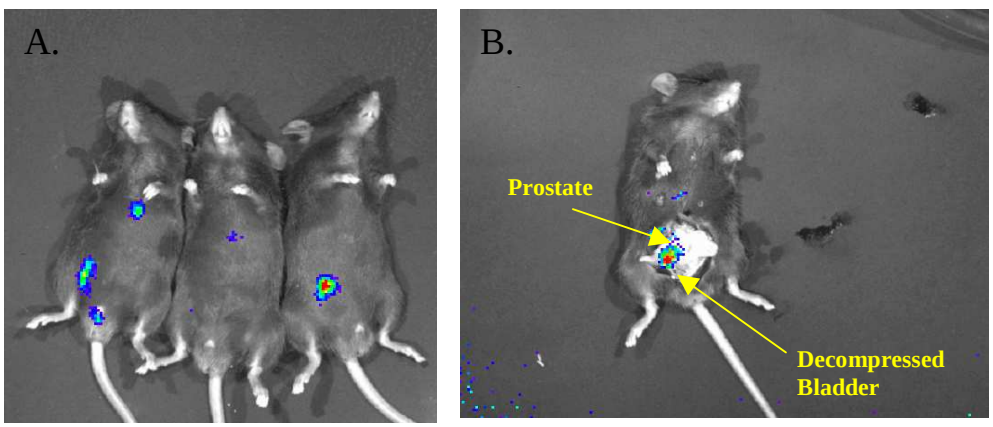
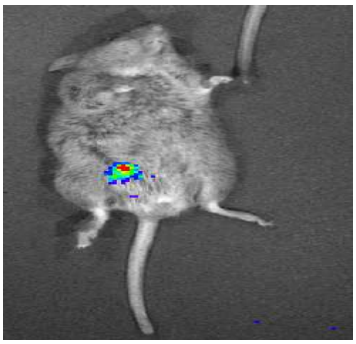


Figure 7: TRAMP-TVA mice intraperitoneally injected with avian virus containing the luciferase gene at 2 days of age were imaged with the CCD camera four months later for stable luciferase activity in the prostate (A). Two/three mice demonstrated signal in the area of the prostate. One of the mice was opened to expose the prostate and confirm the sight of the signal. The bright-red signal was indeed emitted from the prostate.

Trouble-Shooting Approach in Year 2007-2009: As an alternative approach to performing IP injections in immunocompetent adult mice, we bred the TVA mice into a SCID background. We hypothesized that creating an immunodeficient TVA strain will likely enable the following; 1) efficient IP infection of TVA+ tissues in older mice; 2) multiple rounds of infection within a single mouse. After the TVA-SCID crossbred mice were injected with TVA virus at 1 month of age and imaged with the CCD camera (Fig. 8). Although a definite signal was seen in the pelvis, when the mice were opened to identify the exact anatomic location of the luciferase signal, it was evident that the luminescence was not coming from the prostate. Previous experiments using orthotopic injection in our lab have been successful in luciferase gene transfer in mice regardless of age (see Fig. 4). Therefore, orthotopic injection of luciferase virus in TRAMP-TVA mice may be the best approach to achieve durable gene delivery in older mice.



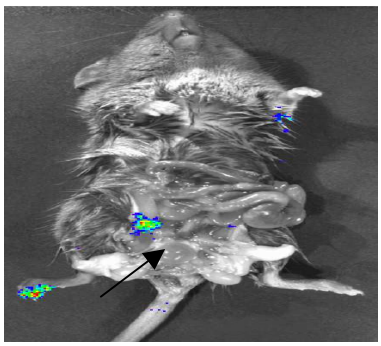


Figure 8. Lack of Infection of prostate epithelium after IP injection of 1-month old SCAI-TVA mice. 3rd generation SCID-TVA mice were injected with RCAS-luciferase virus IP and imaged with the CCD camera. Although luminescence was observed in the pelvis, laporatomy and exposure of the prostate gland (black arrow) demonstrated lack of prosatatic luminescence.

Summary Aim 2: Prostatic infection of TRAMP-TVA mice can be achieved via orthotopic injection. IP injection is successful in TRAMP-TVA pups, but not an efficient means of prostatic infection in older mice, even in immunocompromised mice expressing TVA. Therefore, the orthotopic approach should be used for specific gene transfer into prostate epithelial cells.

Tasks Involved in Accomplishing Aim 3:

1) Creation of RCAS-shRNA vectors targeted against luciferase and SV40 large T antigen
2) In vitro and in vivo analysis of target gene knockdown by RCAS-shRNA viruses

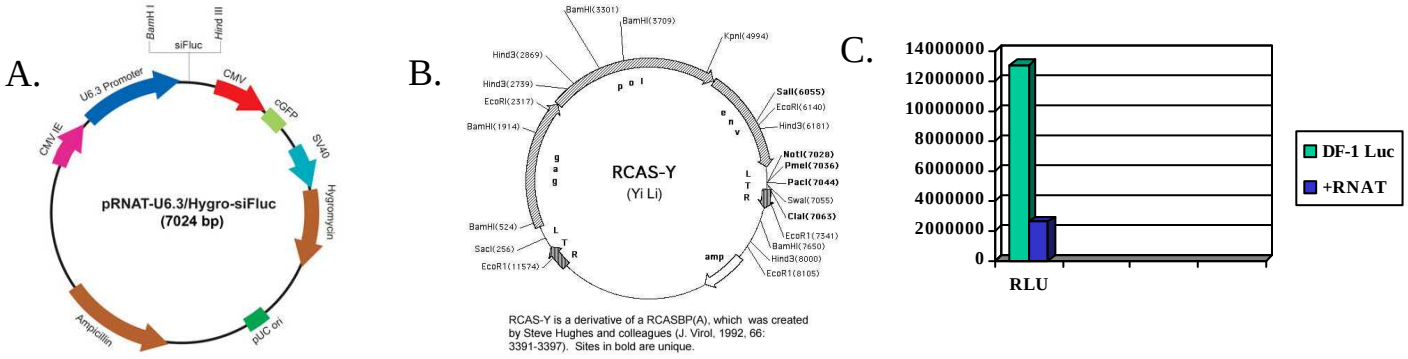


Figure 9: Construction of RCAS vector containing siRNA against Luciferase. A. RNAT vector (Genscript corporation) contains RNAi against luciferase driven by the U6.3 promoter and enhanced with the CMV enhancer. An approximately 2250 bp fragment was amplified from the RNAT backbone and inserted into the Cla and Pac sites (B). Transient transfection of luciferase-infected DF-1 viral producer cells were transiently transfected with RNAT and luciferase activity was measured after 72 hours (C). Greater than 80% reduction in luciferase activity was seen in cells containing RNAT than in controls.

Materials/Methods/Outcomes (Aim 3):
The goal of this aim is to create oligonucleotide sequences will produce shRNAs capable of target gene knockdown and clone them into the avian viral system for in vivo gene transfer into TRAMP-TVA mice. We have acquired a DNA vector that contains shRNAs against luciferase as well as a GFP reporter. Transient transfection of our viral producer cell lines has demonstrated a >80% knockdown in luciferase signal (Figure 9). We have cloned the DNA fragment containing the luciferase shRNA and GFP into our RCAS viral vector. This RCAS-RNAT vector should enable targeting of prostate epithelial cells in TRAMP-TVA mice that have been previously infected with luciferase. We anticipate that with sufficient infection efficiency, we will be able to visualize the emergence of GFP signal and the loss of luciferase. We are in the process of confirming infection efficiency and luciferase knockdown capability in vitro in viral producer cells. Following this, we will perform in vivo infections in TRAMP-TVA mice previously infected with luciferase with prominent, stable activity in the prostate. Depending on the success of the luciferase knockdown experiments, we will go on to produce other shRNAs directed at specific molecular targets, such as SV40 large T antigen, and investigate the in vivo effect on prostate cancer initiation and progression using luciferase or GFP as a co-infected imaging gene.

The timeline initially outlined in the statement of work for the proposed experiments is shown below:

Year 1: Perform mouse breeding to generate cross, analyze mice for timeframe of tumor development and metastasis, confirm TVA expression within prostate cells and tumors, infect mice with luciferase and follow with imaging

Year 2: Continue with imaging experiments; optimize imaging protocol for orthotopic and IP injections, Begin shRNA design and cloning

Year 3: Complete cloning of shRNAs, test in vitro and in vivo, and follow mice with optical imaging

We have accomplished all of the aims listed above.

KEY RESEARCH ACCOMPLISHMENTS:

Generation of TRAMP-TVA Mice
Generation of SCID-TVA Mice
Analysis of Prostate Cancer Initiation in TRAMP-TVA mice
Confirmation of TVA Expression in Prostate Epithelial Cells of TRAMP-TVA mice
Prostate Viral-Mediated Luciferase Gene transfer of TRAMP-TVA mice
Prostate gene transfer into adult SCID-TVA mice
Cloning of Anti-Luciferase shRNA virus

REPORTABLE OUTCOMES: We have a manuscript in preparation. We are well underway to developing this model as a reproducible and efficient method for viral-mediated gene transfer of imaging genes and shRNAs into prostate epithelial cells. We presented a poster at the DOD Prostate Cancer Meeting in 2007 summarizing the results we have achieved thus far.

CONCLUSIONS:

Since initiation of funding, we have accomplished all of the goals outlined in the original statement of work, despite several drawbacks encountered over the past 3 years (difficulty with breeding, change in technical support). We have established a new mouse line that is capable of multiple rounds of viral-mediated gene transfer of imaging and other target genes. We hope that we will be able to apply this model of prostate cancer development and progression for the evaluation shRNA knockdown of a variety of genes.

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